

A comparative and combined approach of isolating genomic DNA from fins of Indian major carps.

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ABSTRACT:

Nondestructive way of collecting tissues and isolation of fare amount of pure genomic DNA using less toxic, inexpensive materials are the prerequisites of molecular characterization of fishes in the studies of population genetics, biodiversity conservation programs and stock management in hatcheries. We describe here such an economic approach to isolate genomic DNA from pectoral fins of Indian major carps with a facility to opt for any one of three inherent DNA extraction methods (1, 2 and 3) of varying quality and quantity output range after a common step of tissue digestion with proteinase K and SDS. Indian major carp rohu (*Labeo rohita*), catla (*Catla catla*) mrigal (*Cirrhinus mrigala*), calbasu (*Labeo calbasu*) and one of crossbreed relative of *L. rohita* and *C. catla* were taken for this experiment. Starting with 1 cm² fin sample range of DNA amounts (70-160 µg) recovered using the option of method 1 were 1.8 to 2.0 times more than that (36.4-88.4 µg) of using the option of method 2 and 3.4 to 6.4 times more than that (19.2-26.5 µg) of using the option of method 3. Quality of DNA recovered from all these three methods were at par for genome analysis studies like PCR amplification of microsatellite markers (here for locus MFW 1 of *Cyprinus carpio*).

Keywords: DNA extraction, Indian major carps, Silica powder, NaCl-Extraction, PCR, microsatellite marker.

INTRODUCTION

With the increasing emphasis on biodiversity issues, population genetics and management of cultured fishes using molecular biology techniques has gained pivotal importance in aquaculture studies (Ferguson, 1995).

High consumer preference and composite culture potential in Indian subcontinent have made *Labeo rohita* Hamilton, *Catla catla* Hamilton and *Cirrhinus mrigala* Hamilton, most preferred carps that contributed 80 % of national annual production (FAO Yearbook of Fishery Statistics, 2005). Owing to the very compatible genomic structure in these carps (Zhang *et al.*, 1991), when bred together in a relatively congregated condition, natural hybrids are frequently encountered that necessitates regular monitoring using genome analysis to determine probable genetic variation.

The procurement of high quality genomic DNA for use in these population genetic studies is the primary concern for researchers. Most if not all genomic DNA extraction protocols required the use of liquid nitrogen and/or freeze-drying (lyophilization) of the tissue for the initial grinding which were not that easy to obtain in every part of the world.

By minimizing the number of steps in traditional DNA extraction procedure (Blin & Stafford, 1976) various simpler procedures had been introduced like salting out method (Miller *et al.*, 1988), microoven-based method (Banerjee *et al.*, 1995), silica-guanidinium thiocyanate method (Carter & Milton, 1993), boiling method (Valsecchi, 1998) and Chelex based extraction method (Walsh *et al.*, 1991) using invasive techniques to procure mostly blood samples.

Without sacrificing the fishes, DNA was obtained using the same invasive procedure from muscle (Hilsdorf, & Caneppele, 1999) or blood samples (Cummings & Thorgaard, 1994; Martinez *et al.*, 1998) but yield was too low.

Non invasive sampling procedures from teleost fishes especially Indian major carps of cyprinidae family are very limited (Yue & Orban, 2001; Wasko *et al.*, 2003) to be used in bulk DNA preparations.

We described here a simple protocol for extraction of sufficient quantity of genomic DNA from Indian major carps. Starting with a little piece of fin and a common step to get total tissue lysate this procedure enables to opt for any one of the three downstream modalities to get DNA of specific quality and quantity of choice or to do DNA extractions using all the three methods side by side to get genomic DNA with varying quality range at a time.

MATERIALS AND METHOD

PROCUREMENT AND PRESERVATION OF FISH FINS

Pectoral fins of desired size (1 cm²) were clipped off using scissors from five Indian major carps that were collected live from different hatcheries and local markets of Ranchi (Jharkhand) and Kolkata (West Bengal) of India. Fin clips were washed thoroughly in water, soaked in tissue paper and suspended in >98% ethanol to store in -20° C for future use in DNA isolation. Three different methods of DNA isolation with common initial step adopted from the modified salt extraction procedure with NaCl (Aljanabi & Martinez, 1997) were tested to compare the efficacy with respect to the amount and purity of the extracted DNA samples.

DNA ISOLATION COMMON STEP OF THREE DIFFERENT PROTOCOLS

Fin samples were washed in distilled water and dissolved in 930 µl of lyses buffer (50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 100 mM NaCl), containing 1% SDS and 150 µg mL⁻¹ of proteinase K (Sigma) in a 2 ml Eppendorf microtubes. After thorough mixing the tubes were placed in temperature regulated water bath at 50°C from 8-12 hrs with intermittent vortexing for 30 seconds. Progression of tissue lyses were checked time to time. To assess the RNase treatment result, one set of samples were treated with 100-200 µg mL⁻¹ of RNase (Sigma) and incubated further for one hr in 37°C. Both sets of samples were centrifuged for 10 minutes in 12500 rpm at room temperature. Supernatants were transferred and distributed (300 µl each) in three Eppendorf microtubes for further treatments of three different protocols to be followed. Total amount of supernatant could also be taken for any one of the three following methods.

METHOD 1: MODIFIED NaCl EXTRACTION

300 µl of 5M NaCl solution was added with the 300 µl of proteinase K digested tissue lysate and mixed well by inverting several times. Mixture was then centrifuged at 12000 rpm for 10 minutes to take out the supernatant. This step was repeated to clear the supernatant. Then added 360 µl prechilled ethanol to precipitate the DNA. The precipitated DNA appears cloudy which disappears and turned into DNA clump after proper mixing. Tubes were kept in -20°C for at least one hr and centrifuged at 12000 rpm, 4°C for 10 minutes. The DNA pellet were washed with 350 µl of 70% ethanol, air dried in laminar hood and suspended in 50 µl TE (10 mM of Tris pH 8.0 and 1 mM of EDTA) to be stored in -20°C.

METHOD 2: MODIFIED PHENOL-CHLOROFORM EXTRACTION

300 µl of proteinase K digested tissue lysate was first extracted twice with 150 µl each of TE saturated phenol and then twice with 150 µl each of chloroform mixed with isoamyl alcohol (24:1) to purify the DNA. Clear upper phase was carefully taken out and added equal volume of 3M Na-acetate solution plus 2.5 volume of cold ethanol. Solution mixed by gently inverting the tubes up and down for few times and kept at least one hr in -20°C for complete precipitation of DNA. Rests of the steps were similar to method 1.

METHOD 3: MODIFIED CHAOTROPIC SALT-SILICA POWDER EXTRACTION

300 µl of proteinase K digested tissue lysate was added with cold 3M sodium acetate solution (3 molar with respect to sodium and 5 M with respect to acetate) and mixed well without vortex. Solution was kept in ice for 5 minutes and centrifuged at 12000 rpm for 5 minutes.

Clear supernatant was taken out and mixed well with 3 volumes (approx. 1350 µl) of chaotropic salt solution (HiMedia). Then 25 µl of silica powder solution (HiMedia) was added, mixed well and kept in room temperature for 10-15 minutes with occasional up and down movements of the tubes. Mixture was then centrifuged at 12000 rpm for 10 seconds and supernatant was discarded. Pellet was washed twice with 1250 µl of washing buffer (HiMedia) by dissolving the DNA-Silica powder pellet pipetting back and forth with tips followed by brief spin at 12000 rpm). After taking out the supernatant of the last wash, centrifuged the tube again to remove the traces of ethanol that was left still. Air dried the pellet in laminar hood for less than 5 minutes and suspended in 50 µl TE and incubated 5 minutes in -55°C water bath for elution of DNA from silica surface before centrifuging at 12000 rpm for 50 seconds. Took out the supernatant DNA solution and repeated the elution step for second time to 10-20% further recovery of DNA. Pooled up total DNA was finally dissolved in 50 µl of TE.

VERIFICATION AND QUANTIFICATION OF EXTRACTED DNA

Genomic DNA quality was checked by horizontal electrophoresis in 0.8% agarose gel in 1X TBE buffer. Gel was stained with ethidium bromide and photographed using Gel Doc System (BioRad). The quantity and quality of extracted DNA were also measured by the absorbance at 260 nm and ratio of absorbance of 260/280 nm or 260/230 respectively in spectrophotometer (Eppendorf) using 50 fold dilutions of the DNA samples with distilled water.

PCR AMPLIFICATION

Genomic DNA extracted by all three modified methods from fins of major Indian carps taken for this experiment were tested for PCR amplification using a common specific primer for the microsatellite molecular marker of *Cyprinus carpio* Linnaeus of locus MFW 1 (Lal et al., 2004) having CA repeat motif within approximately 168 bp stretch of DNA. PCR were carried out in 10 µl reaction volume containing 30-50 ng template DNA, 10 uM of each primer, 1x reaction buffer (NEB), 0.2 mM of each of dNTP (NEB) and 0.5U of taq DNA polymerase (NEB) using Gradient Mastercycler of Eppendorf under the following conditions: The PCR began with a 94°C enzyme-activating step for 5 min, followed by a touchdown program (94°C denaturing step for 30 s followed by initial annealing temperature of 70°C, subsequently run down to 54°C at 1°C/cycle, 72°C extension step for 1 min), followed by a uniform three-step amplification profile (94°C denaturing step for 30 s, 54°C annealing step for 30 s, 72°C extension step for 1 min) for another 23 cycles, then 72°C for 10 min, and finally held at 4°C.

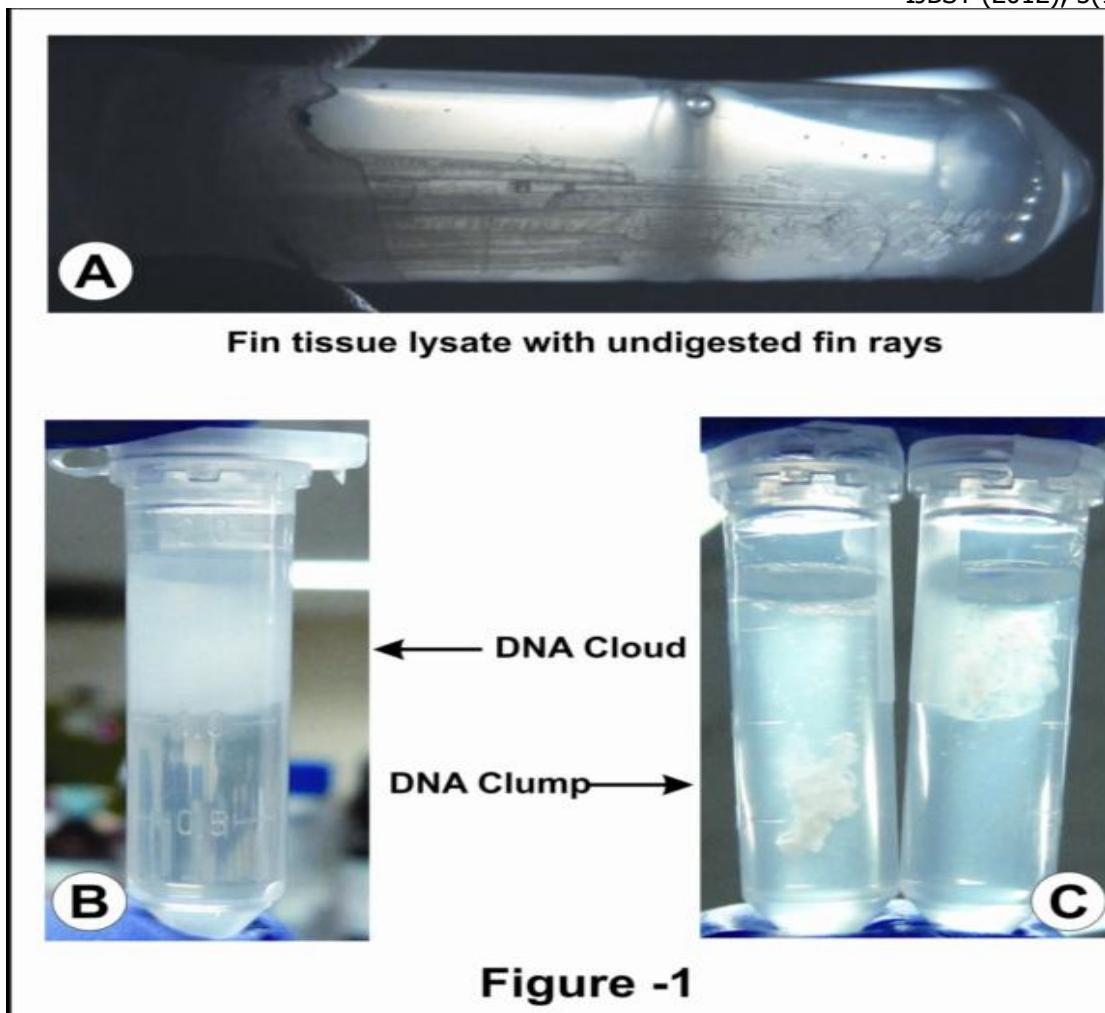
**Figure -1**

Fig. 1 A Totally digested fin tissues with undigested fin rays. B, Formation of DNA cloud in the step immediately after adding the cold ethanol of Method 1. C, DNA clump formed after mixing the ethanol by inverting the tubes for 2-3 times.

Table I: Concentration of Nucleic Acid in 50 µl TE with corresponding $A_{260/280}$ values

Fishes Taken	Method 1		Method 2		Method 3	
	Nucleic acid conc. in ng/µl	$A_{260 / 280}$ (mean \pm S.E*) (average: 1.72 \pm 0.015)	Nucleic acid conc. in ng/µl	$A_{260 / 280}$ (mean \pm S.E*) (average: 1.91 \pm 0.046)	Nucleic acid conc. in ng/µl	$A_{260 / 280}$ (mean \pm S.E*) (average: 1.89 \pm 0.014)
<i>L.calbasu</i>	3161	1.77 \pm 0.026	1654	1.86 \pm 0.030	482	1.91 \pm 0.015
<i>C. catla</i>	2098	1.68 \pm 0.020	1038	1.85 \pm 0.030	436	1.87 \pm 0.030
<i>C. mrigel</i>	1727	1.71 \pm 0.026	880	1.90 \pm 0.041	411	1.89 \pm 0.030
<i>L. rohita</i>	1399	1.75 \pm 0.011	728	2.10 \pm 0.102	385	1.85 \pm 0.025
Crossbreed	3196	1.73 \pm 0.041	1768	1.88 \pm 0.023	530	1.93 \pm 0.026

* number of observations (n) = 3

Table I shows Comparison of quality ($A_{260 / 280}$) and quantity (concentration multiplied by volume) of genomic DNA isolated by three different methods from four natural Indian major carps and one Crossbreed of *L. rohita* and *C. catla*. From each fish a single fin tissue block of 1 cm² was digested and distributed equally into three microfuge tubes to be extracted using three different methods. Samples taken were in triplicate. Extracted DNA by all three methods was dissolved in same amount of TE (50 µl) and diluted 50 fold to be analyzed in spectrophotometer.

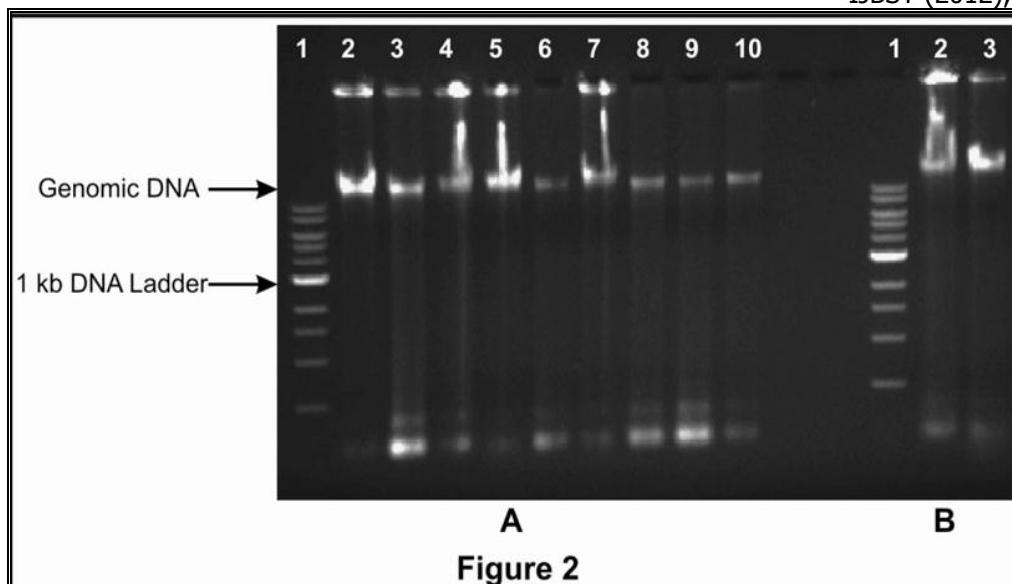


Fig. 2A RNase untreated genomic DNA bands of *L. rohita*, *C. catla* and *C. mrigala* as three representative fishes of Indian major carps in 0.8% agarose gel electrophoresis with equal volumes of DNA solutions loaded from each of three methods tested. Lane 1, 1kb DNA ladder, lane 2-4, DNA from method 1, lane 5-7 DNA from method 2, lane 8-10 DNA from method 3, B; Lane 1, 1kb DNA ladder, 2 and 3 RNase untreated genomic DNA bands from calbasu fin with attached myotome tissue and same fish fin without myotome tissue respectively.

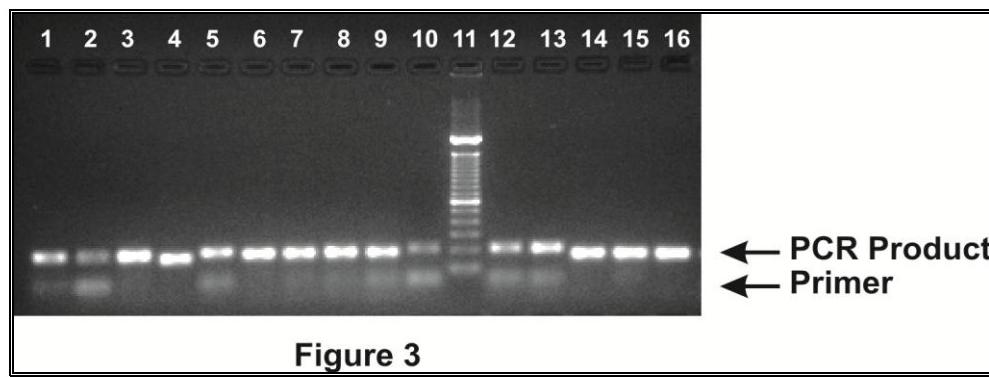


Fig. 3 PCR amplification using both RNase treated and untreated DNA samples as template from all three methods tested with microsatellite primer of MFW 1 of *Cyprinus carpio* in all the five Indian major carps. Set of three lanes of 1-3, 4-6, and 7-9, 10 and 12-13, 14-16, represent PCR products of five Indian major carps taken in this experiment. First two lanes and the last lane of the set of 3 lanes represent as RNase untreated and treated DNA respectively used as template.

Table II: Comparison of quantity and quality of DNA extracted by method 1 of fin samples with or without attached supporting myotome muscles

Fish Fin Type	DNA conc. in ng/uL	A _{260 / 280}	A _{260 / 230}
<i>L. calbasu</i> Fin with myotome	6112	1.71	1.47
<i>L. calbasu</i> Fin only	3860	1.77	1.73
Crossbreed Fin with myotome	7139	1.57	1.09
Crossbreed Fin only	3786	1.71	1.68
<i>C. catla</i> Fin with myotome	6491	1.63	1.12
<i>C. catla</i> Fin only	3580	1.71	1.65

Table II shows Comparison of quality ($A_{260 / 230}$ and $A_{260 / 280}$) and quantity (concentration multiplied by volume) of genomic DNA isolated exclusively by method 1 of myotome attached and unattached fin tissues from two natural Indian major carps and one Crossbreed of *L. rohita* and *C. catla*. Fin tissues, whether with attached myotome or not, taken in this case were of same weight and DNA was extracted using only method 1 after the common step of tissue digestion. Extracted DNA was dissolved in same amount of TE (in 150 μ L TE) and diluted 50 fold to be analyzed in spectrophotometer.

RESULTS

Progression of dissolution of fin tissues in the backdrop of undigested fin rays was clearly visible at different times and final stage of lyses was documented in Fig 1A. Amount of Genomic DNA isolated by Method 1 was proportional to the density and amount of DNA cloud formed (Fig 1B) in the step immediately after adding the cold ethanol and also to the amount of DNA clump (Fig 1C) formed in the next step of mixing the ethanol by inverting the tubes for two to three times.

Intensity of genomic DNA bands in gel electrophoresis applying equal volumes of DNA solutions from each of three methods tested showed concentration in the order of maximum in method 1 to minimum in method 3 corroborated well with the amount of DNA as deducted from the spectrophotometer reading at 260 nm (Table I). The quality of the isolated genomic DNA were good enough to allow PCR amplification of simple sequence repeat polymorphisms without further purification but the quantity varied significantly (highest and lowest being 3196 and 385 ng / μ l TE by method 1 and method 3 respectively).

Amount of DNA recoveries from all five Indian major carps were in the range of 70 - 160 μ g by method 1, 36.4 – 88.4 μ g by method 2, and 19.2 – 26.5 μ g by method 3 in total 50 μ l TE (Table I).

Presence of negligible amount of contaminant RNA in isolated DNA samples using any of the three methods tested without RNase treatment was evident either from the agarose gel electrophoresis picture (Fig 2A) or from below 2.0 average $A_{260/280}$ values of DNA solution (Table I).

PCR amplification using both RNase treated and untreated DNA samples as template from all three methods tested with microsatellite primer of MFW 1 of *Cyprinus carpio* worked equally well in all the five Indian major carps (Fig 3).

Genomic DNA bands in agarose gel (Fig 2A) from method 3 were found most clear corroborated well with an average value of 1.89 for $O.D_{260/280}$ but bands from method 2 with certain amount of impurities were bit inconsistent with apparently normal 1.91 average $O.D_{260/280}$ value (Table I).

When total amount of tissue lysate from common step of three methods was tested with only method 1, hitherto proved most efficient one for maximum recovery, up to 7139 ng/ μ l of DNA could be extracted in 150 μ l TE from fin samples with attached myotome muscles. Fins with attached supporting myotome muscles when compared for DNA extraction with only fin samples without myotome using the same method 1, though 1.5-2.5 fold more amount of DNA was extracted but of inferior quality as evidenced either by 0.3 to 0.7 less of 260/230

nm absorbance values than 1.8 of pure form of DNA in TE (Table II) or by the quality of DNA bands of lane 2 and 3 in figure 2B.

DISCUSSION

The result of present investigation reveals that the time required for digestion of different fins by proteinase K was not uniform. One could calculate the time for any experimental set with different fish samples by comparing the final lysis stage as shown in Figure 1A. One could even assure of keeping right track with the extraction procedure of method 1 by simply observing the formation of DNA cloud followed by DNA clump.

Apparently unclear genomic DNA bands of method 2 with inconsistent spectrophotometric data of $O.D_{260/280}$ might either because of contaminant proteins rich in aromatic amino acids which absorb light at 280nm or contaminating phenol with peak absorption at 270nm that singly or in combination threw off quantification values towards 1.8 - 2.0 though in reality it would have been much shifted.

The method 1 did not require corrosive reagents of method 2 like phenol and cumbersome transferring process of aqueous upper phase from the ever interfering bottom phase of organic solvent that always tend to come up to be mixed with the clear phase in spite of every precaution taken to render $A_{260/280}$ values of DNA solution a bit confusing. This method 1 did not require the costly products of method 3 like Silica powder with related chaotropic salt and washing solutions either and the repeated steps of washing DNA-Silica powder pellet before elution. Still the astounding amount and the apparent good quality of the DNA isolated by this method 1 were high enough to do thousands of PCR based reactions for fish microsatellite amplification. As PCR amplification using both RNase untreated and treated DNA samples from all three methods with microsatellite primer of MFW 1 of *Cyprinus carpio* worked exquisitely the RNase treatment step could reasonably be omitted from this method 1 to further cut down the price for bulk extractions of fish DNA for the studies of population genetics and stock management.

DNA isolated from method 3 though appeared as purest form with only certain amount of RNA contamination, the recovery was alarmingly less (3.4 to 6.4 fold) than method 1. This method 3 may only be tried when need for less quantity but highly purified DNA overshoots the demand for large quantity of DNA of moderate purity usable for routine experiments of population genetics.

The fact of getting inferior quality DNA following high throughput DNA extraction method 1 from fin samples with attached myotome (Fig 2B) can be explained by the presence of organic compounds like polysaccharide spouted from the extensive tissue lyses step by proteinase

K and SDS which absorbs wavelength 230 nm mostly to put the $A_{260/230}$ values 0.3 to 0.7 down (Table II). Thus it was logical not to include myotomes while collecting the fin samples.

So we concluded by providing here a PCR amplifiable genomic DNA extraction protocol with an initial common step and three optional methodologies from fresh or ethanol preserved fish fin samples from Indian major carps for the first time that may be applicable for other ray-finned fishes. Three separate modified methodologies with difference in simplicity and speed would separately ensure recovery of DNA with varying quality and quantity necessary to address a specific situation of downstream genetic analysis of fishes. The qualities of DNA isolated in all the three modified methods tested were high enough to allow at least PCR amplification of simple sequence repeat polymorphisms without further purification.

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